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Research Paper

Multi-Strain Probiotics Inhibit Cardiac Myopathies and Autophagy to Prevent Heart Injury in High-Fat Diet-Fed Rats

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Abstract

High-fat diets induce obesity, leading to cardiomyocyte fibrosis and autophagy imbalance. In addition, no previous studies have indicated that probiotics have potential health effects associated with cardiac fibrosis and autophagy in obese rats.

This study investigates the effects of probiotics on high-fat (HF) diet-induced obesity and cardiac fibrosis and autophagy in rat hearts. Eight-week-old male Wistar rats were separated randomly into five equally sized experimental groups: Normal diet (control) and high-fat (HF) diet groups and groups fed a high-fat diet supplemented with low (HL), medium (HM) or high (HH) doses of multi-strain probiotic powders. These experiments were designed for an 8-week trial period. The myocardial architecture of the left ventricle was evaluated using Masson's trichrome staining and immunohistochemistry staining. Key probiotics-related pathway molecules were analyzed using western blotting. Abnormal myocardial architecture and enlarged interstitial spaces were observed in HF hearts. These interstitial spaces were significantly decreased in groups provided with multi-strain probiotics compared with HF hearts. Western blot analysis demonstrated that key components of the TGF/MMP2/MMP9 fibrosis pathways and ERK5/uPA/ANP cardiac hypertrophy pathways were significantly suppressed in probiotic groups compared to the HF group. Autophagy balance is very important in cardiomyocytes. In this study, we observed that the beclin-1/LC3B/Atg7 autophagy pathway in HF was increased after probiotic supplementation was significantly decreased. Together, these results suggest that oral administration of probiotics may attenuate cardiomyocyte fibrosis and cardiac hypertrophy and the autophagy-signaling pathway in obese rats.

Key words: High-fat diet; Obesity; probiotics; Autophagy.

Introduction

Obesity is a metabolic syndrome that always leads to cardiovascular diseases (CVDs). Diseases occur in obese patients who have hyperlipidemia, adipose tissue inflammation and oxidative stress, insulin resistance, lipid metabolic disorder and carcinogenesis [1-6]. These factors are all important

pathological phenomena. Obesity is highly associated with increased CVD [7]. The modern first world diet makes it easy to intake high-fat foods, often leading to obesity, hypertension and insulin resistance, which are common and detrimental health problems [8]. The obesity-induced tissue damage mechanism in an animal model fed a high-fat diet involves caspase-dependent apoptosis resulting in cardiac dysfunction [9]. In genetically obese mice, the apoptotic response in cardiomyocytes was obtained from mitochondrial damage that increased and down-regulated the survival rates [10].

A previous study indicated that left ventricular hypertrophy is highly associated with obesity [11]. Stress promotes cardiac hypertrophy. Exercise and pregnancy induce reversible physiological cardiac hypertrophy, whereas inflammation, hypertension and myocardial injury result in irreversible pathologic increases in hypertrophy [12]. The IL-6 signaling pathway is associated with cardiomyocyte remodeling. IL-6 and IL-6 receptor activation is induced by signaling pathways such as the mitogen-activated protein kinase 5 (MEK5) pathway, the signal transducer and activator of transcription 3 (STAT3) pathway and the MAPK extracellular signal-regulated kinase (ERK) pathway. The activation of MEK5/ERK5/STAT3 signaling pathways was found to promote cardiac myocyte eccentric hypertrophy, which is one type of pathologic hypertrophy [13-15].

Our recent research showed that a high-fat diet induced cardiac apoptosis [16]. Previous papers indicated that apoptosis is a familiar mechanism for removing redundant cells, and it always results in down-regulated cell proliferation. In addition, apoptosis plays an important role in cardiac injury pathogenesis [17, 18]. After cardiac apoptosis, the gap in cardiomyocyte arrangement is filled by fibroblasts [19]. Cardiac fibrosis is the last stage in the heart failure process [20], leading to severe heart dysfunction. External pressure or pathological pressure for the heart may lead to cardiac fibrosis. Although cardiac fibrosis is an important stage in the progression of heart disease, the mechanisms underlying fibrosis development and progression are unclear [21]. Our previous study revealed that transforming growth factor beta (TGF-β) activated the downstream pathway, increased matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) expression and promoted transcription factor specificity protein 1 (SP1) up-regulation [22]. Moreover, a recent paper indicated that the fibrosis pathway up-regulates SP1 leading to cardiac remodeling and fibroblast proliferation, and also induces connective tissue growth factor (CTGF) expression. Another study also indicated that CTGF activation is associated with cardiac fibrosis and cardiac remodeling [23].

Autophagy as an energy balance mechanism also implicated in degradation and recycling of damaged organelles, proteins and carbohydrate (glycogen), may be important for metabolism system regulation [24]. Other studies indicated that when the degradation and recycling mechanism is overloaded, the cells die by autophagy-dependent cell death [25, 26]. Autophagy is a multi-step process involving many key proteins. Beclin-1 is a key protein in the early stages that promotes the autophagy process [27], and LC3BI interacts with ATG7 to induce the maturity of autophagosomes [28]. The role of autophagy is different in different cells and situations. One study indicated that the pathophysiology of obesity is highly associated with increased autophagy in adipose tissue [29].

Probiotics have been reported to promote metabolism and adjust immune function and gastrointestinal health. Additional evidence has indicated that probiotics exert a biologic effect on the heart [30-35]. There are some studies that indicated that probiotics regulated autophagy expression [36-38]. In this study, we used a high-fat diet to set up an obesity animal model to investigate if oral probiotic administration prevents obesity-induced heart fibrosis and autophagy imbalance.

Materials and methods

Animal model and probiotic suspensions

Wistar rats were purchased from the National Laboratory Animal Centre in Taipei, Taiwan. Animals were housed individually in a temperature and humidity controlled environment at 25±2˚C and 50±5% humidity. The rats were maintained on a 12-h dark-light cycle with lights on from 8:30 AM to 8:30 PM. They were provided chow pellets AIN-76 purchased from Young Li Trading Co. Ltd., Taipei, Taiwan and water *ad libitum* during an eight-week experiment period. The rats were randomly divided into five groups: A normal control group (Control), a high-fat (HF) diet group fed AIN-76 with 15.47% butter powder, and three groups given different doses of multi-strain probiotics with AIN-76 with 15.47% butter powder. The three groups were as follows: low (78 mg/kg/day, 4.18×10^5 CFU/ml, HL), medium (390 mg/BW/day, 4.22×10^6 CFU/ml, HM) or high (1950 mg/kg/day,4.48×10^7 CFU/ml, HH). Multi-strain probiotic powder (*Lactobacillus rhamnosus: Pediococcus acidilactici*: *Bifidobacterium adolescentis*; 1: 1: 1) was produced by freeze-drying and obtained from New Bellus Enterprise Co., Ltd (Tainan, Taiwan). The

entire experiment was performed according to the NIH Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee of China Medical University, NO.101-263-B.

Tissue protein extraction

Heart tissue extracts were collected from the rats by homogenizing the left ventricular samples in lysis buffer (50 mM Tris-HCl, 0.5% NP-40, 250 mM NaCl, 5 mM EDTA and 50 mM NaF). The homogenate samples were placed on ice for 30 min with vortex every 5 min and then centrifuged at 12500 rpm for 30 min. The supernatants were collected and stored at -80°C for use in further experiments.

Western blot analysis

The heart tissue protein concentrations were measured using Lowry's protein assay method. The heart tissue protein samples were subjected to 13.5%, 10% or 8% SDS–PAGE at a constant voltage of 70 V. The proteins separated via SDS–PAGE were transferred onto polyvinylidene difluoride membranes (EMD Millipore Life Sciences) using a current of 100 V for 60 min. The membranes were incubated in blocking buffer (5% fat-free milk in Tris-buffered saline buffer) for 1 hr and then incubated in primary antibodies against specific proteins: MMP2(sc-13595, Santa Cruz Biotechnology), MMP9 (sc-6841, Santa Cruz Biotechnology, Dallas, Texas, USA), SP1(sc-59-G, Santa Cruz Biotechnology, Dallas, Texas, USA), TGF-β (sc-31609, Santa Cruz Biotechnology, Dallas, Texas, USA), Beclin-1 (#3738, Cell Signaling Technology, Maryland, USA), LC3B (#2775, Cell Signaling Technology, Maryland, USA), ATG7 (#2631, Cell Signaling Technology, Maryland, USA), ERK5 (sc-1284, Santa Cruz Biotechnology, Dallas, Texas, USA), MEK5 (sc-9320, Santa Cruz Biotechnology, Dallas, Texas, USA), uPA (sc-14019, Santa Cruz Biotechnology, Dallas, Texas, USA), ANP (sc-20158, Santa Cruz Biotechnology, Dallas, Texas, USA) and tubulin (sc-5286, Santa Cruz Biotechnology, Dallas, Texas, USA). The antibodies were diluted 1:1000 in Tris-buffered saline buffer overnight. Horseradish peroxidase-labelled secondary antibodies were applied, and the results were obtained using a Fujifilm LAS-4000 mini imager (GE Healthcare Life Science).

Masson's trichrome staining

The rat heart tissue from each group was stored in 10% formalin for 2 weeks, dehydrated using an alcohol gradient (75%, 85%, 90%, and 100% alcohol, 5 min each) and embedded in paraffin wax. The 0.2 μm-thick paraffin sections were then sliced from these paraffin-embedded tissue blocks. The tissue sections were de-paraffinized via immersion in xylene (3 times, 5 min each) and rehydrated using an alcohol gradient (100%, 90%, 85%, and 75% alcohol, 5 min each). Biopsy samples were then stained using Masson's trichrome stain to investigate heart morphologic and fibrotic changes; blue staining represented collagen accumulation. The results were obtained using an OLYMPUS microscope.

Immunohistochemistry staining

Heart sections from the Wistar rats were stained through the use of an immunohistochemistry kit (no.760-700, Roche Life Science, Indianapolis, USA), and the target protein was identified by brown color. As primary antibodies, LC3B Ab (#2775, Cell Signaling Technology, Maryland, USA) were used.

Statistical analysis

The data are presented as the means ± SD from three independent experiments. Statistical analysis was performed via one-way analysis of variance. Student's t-test was applied for paired samples.

Results

Masson's trichrome stain assay in heart tissue sections

Whole heart tissue section samples from the high-fat diet-induced obese rats were stained using Masson's trichrome. The results showed that the heart cell arrangement was disordered with high collagen accumulation (blue color) in the HF group compared with the control group (Figure 1A and B). After supplementation with the different dosages of multi-strain probiotics, the heart arrangement was rescued with significantly decreased collagen accumulation (Figure 1C-D). The multi-strain probiotics decreased the collagen accumulation in a dose-dependent manner compared with the HF group (Figure 1B-D).

Evaluation of the expression of cardiac fibrosis protein markers in the hearts of rats fed a high-fat diet with or without different doses of multi-strain probiotics

The change in the fibrosis protein marker levels in rat hearts were investigated using western blotting assay of heart tissue protein extracts. Figure 2A shows that MMP2 and TGF-β expression up-regulated in the HF group after supplementation with different doses of multi-strain probiotics. The expression of MMP2 and TGF-β decreased in a dose-dependent manner. The fibrosis-related transcription factors SP1 expression in the hearts from the high-fat diet group was significantly increased when compared to the control group. SP1 expression was down-regulated in

the low-, medium- and high-dose multi-strain probiotic groups compared with the control group. Moreover, quantification of the Western blot data showed that multi-strain probiotic supplementation prevented high-fat diet-induced heart fibrosis due to the down-regulation of the TGF-β/MMP2/SP1 pathway in a dose-dependent manner.

Effect of probiotics on cardiac autophagy early stage-associated protein markers

Previous studies indicated that LC3B and ATG7 play a key role in the early steps of the autophagy molecular pathway [39]. In this research, we observed that rats fed with a high-fat diet induced a change in the autophagy molecular pathway (Figure 3). The results showed that expression of autophagy early step-associated protein markers LC3BI and ATG7 was increased in the HF group. Oral intake of the multi-strain probiotics in high-fat diet-fed rats decreased the expression of LC3BI and ATG7 in each different dose in the multi-strain probiotic groups (HL, HM and HH, Figure 3A) and in a dose-dependent manner. Importantly, we revealed that autophagy changed in the early steps in obese rat hearts, but after oral intake of the multi-strain probiotics, only the LC3BI and ATG7 pathways were significantly decreased. Moreover, the expression of beclin-1 increased in the HF group (Figure 3A), but there was no significant difference after supplementation with multi-strain probiotics. These findings suggested that a high fat diet affected autophagy in the early stages and supplementation with multi-strain probiotics decreased the high-fat diet-induced autophagy imbalance regulated by LC3BI and ATG7 expression.

Cardiac tissue section IHC staining analysis

To confirm whether multi-strain probiotics decreased high-fat diet-induced autophagy activation by down-regulating LC3B expression, cardiac tissue section samples from the high-fat diet-induced obese rats were stained with IHC staining. The results showed that LC3B was highly expressed in the HF group compared with the control group (Figure 4A and B, brown color). Following treatment with low-, medium- and high-dose multi-strain probiotics, a reduction in the LC3B expression was observed (Figure 4C-E). This result confirmed that multi-strain probiotics regulated the autophagy pathway via inhibiting LC3B expression in a dose-dependent manner.

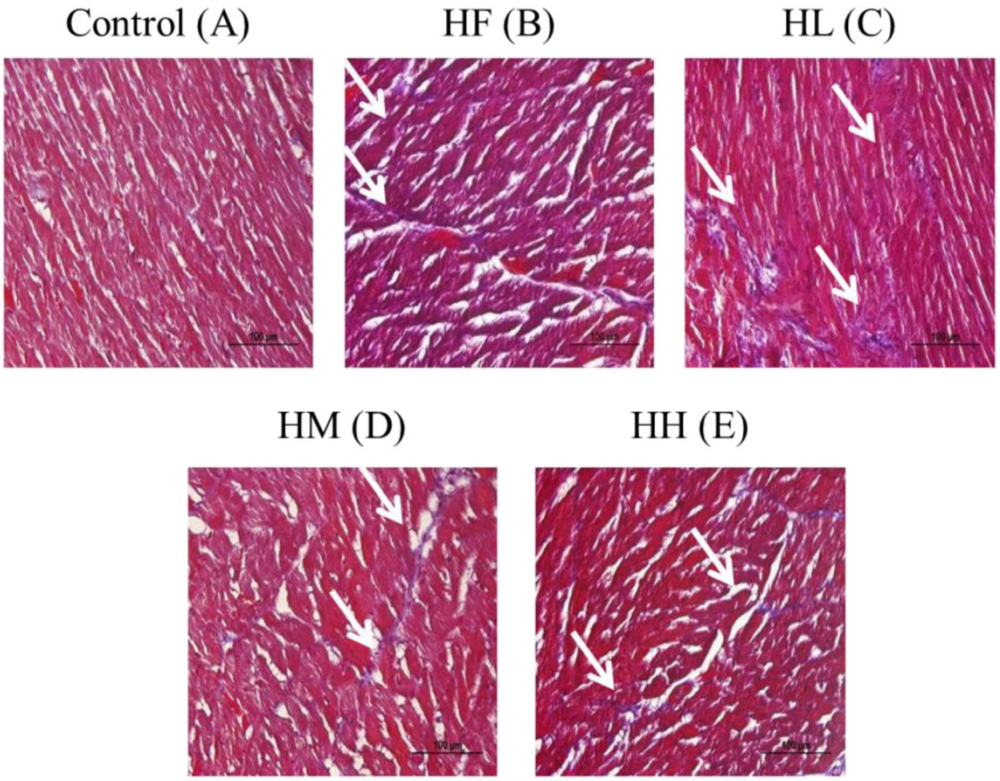

Figure 1. Masson's trichrome staining of cardiac tissue sections. Histopathological analysis of cardiac tissue sections of the left ventricle from the control rats, the high-fat diet-induced obese rats, the high-fat diet-fed rats treated with low dose, medium dose and high dose of multi-strain probiotics. White arrow: site of collagen accumulation.

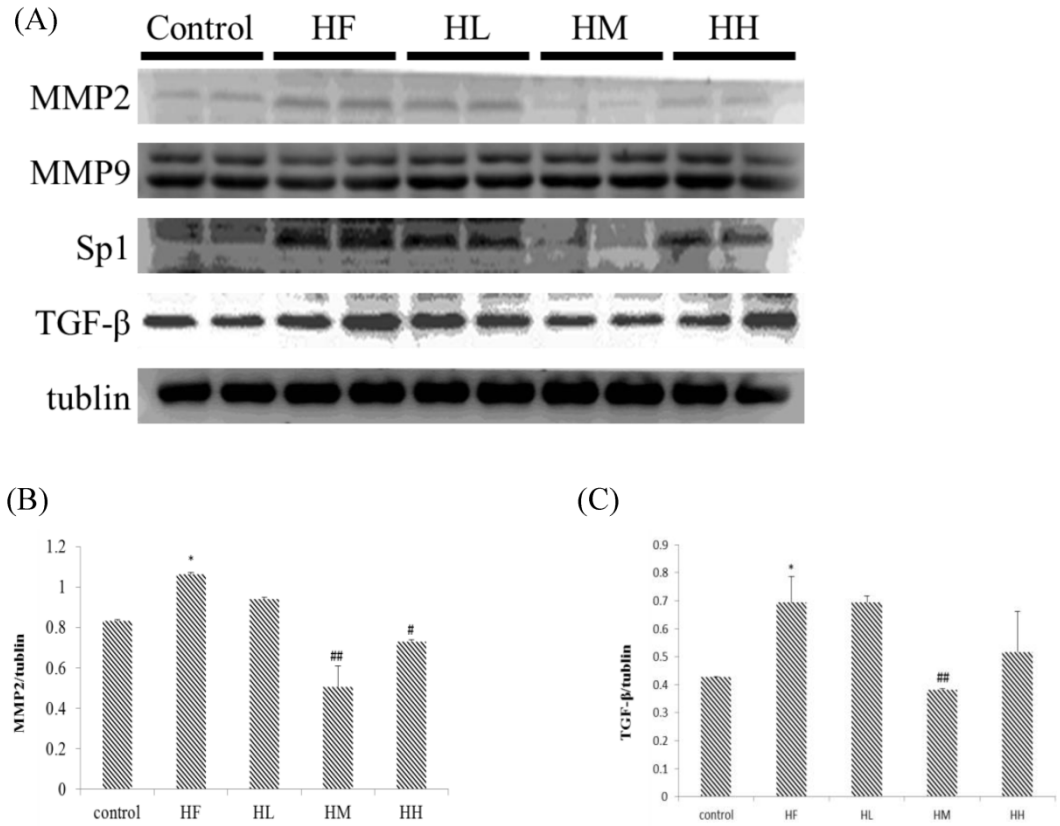

Figure 2. Fibrosis-related protein expression analysis via Western blot. The fibrosis-related protein levels were increased in the left ventricular tissue of high-calorie diet-induced obese rats, whereas treatment with different doses of multi-strain probiotics reduced these fibrosis-related proteins. (A) Western blots for the fibrosis pathway proteins. Quantitative analysis of the expression levels of (B) MMP2 or (C) TGF-β normalized to those of tubulin, #P<0.05, ##P<0.01 or ###P<0.001: the mean values were significantly different from those of the control group.

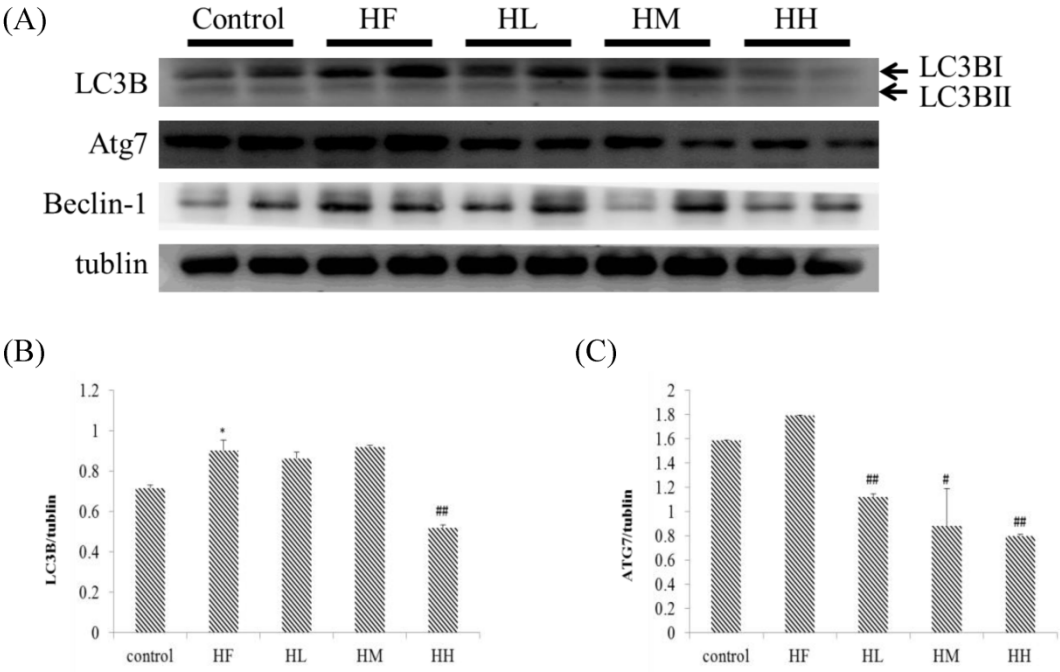

Figure 3. Autophagy early stage-related protein expression analysis via Western blot. The autophagy-related protein levels were increased in the left ventricular tissue of high-calorie diet-induced obese rats, whereas treatment with different doses of multi-strain probiotics reduced these fibrosis-related proteins. (A) Western blots for the fibrosis pathway proteins. Quantitative analysis of the expression levels of (B) LC3B or (C) ATG7 normalized to those of tubulin, #P<0.05, ##P<0.01 or ###P<0.001: the mean values were significantly different from those of the control group.

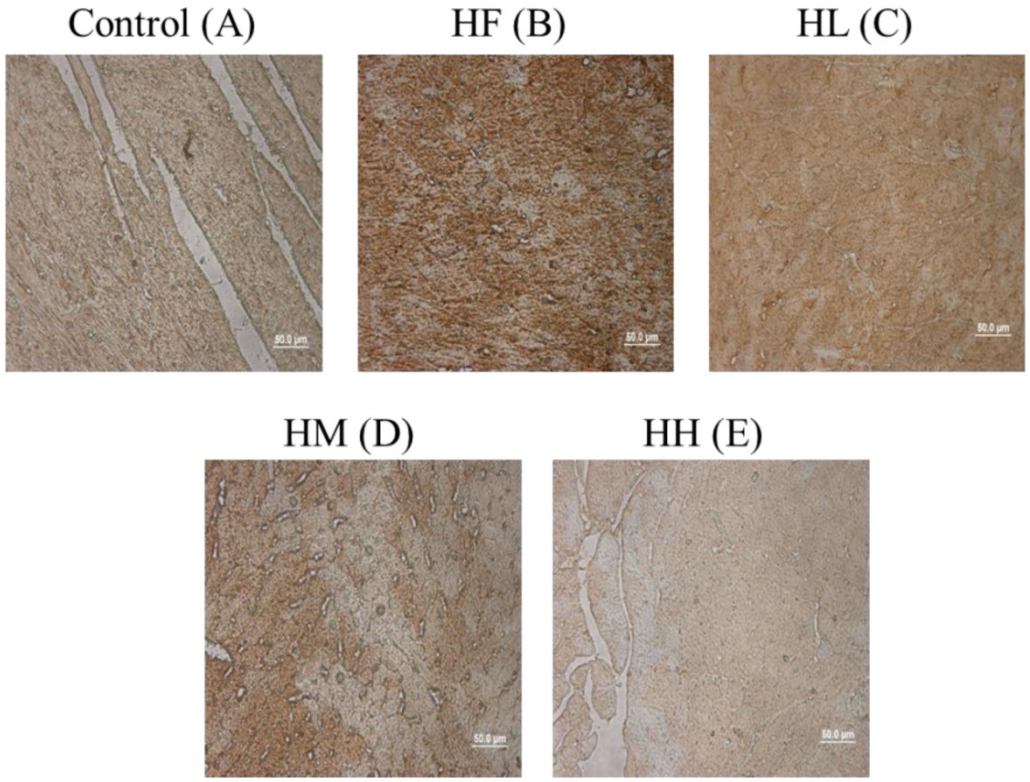

Figure 4. ICH staining of cardiac tissue sections. Histopathological analysis of cardiac tissue sections of the left ventricle from the control rats, the high-fat diet-induced obese rats, the high-fat diet-fed rats treated with low dose, medium dose and high dose of multi-strain probiotics. Brown color: site of LC3B expression.

Multi-strain probiotic effect on the cardiac hypertrophy pathway in the hearts of rats fed a high-fat diet

In this study, we determined if a high-fat diet induced heart injury through promoting cardiac hypertrophy. Figure 5 shows that after feeding a high-fat diet, the expression of upstream protein markers ERK5 and MEK5 in cardiac hypertrophy was increased compared with the control group as determined by Western blot assays. Treatment with different doses of multi-strain probiotics decreased ERK5 and MEK5 expression. Although there was no significant difference between the control group and HF group on uPA and ANP expression, we observed that multi-strain probiotics decreased uPA and ANP expression in different doses (Figure 5). These findings indicate that multi-strain probiotics prevent high-fat diet-induced cardiac hypertrophy.

Discussion

Recent research has determined that probiotics present the following biological effects: decreased free radical injury, anti-inflammation, and inhibition of tumors and the regulation of lipid metabolism [40-43]. Our previous studies showed that supplementation with probiotics promoted cardiac survival through activation of the PI3K/Akt pathway and decreased inflammatory and fibrosis protein expression in the hearts of spontaneously hypertensive rats [44, 45]. Other studies observed that lactic acid bacteria, a probiotic, decreased blood glucose levels and provided a protective effect in ischemic animal heart models [46, 47]. The first world human lifestyle is characterized by the intake of high levels of fat and calories, resulting in obesity. Obesity is one of the metabolic diseases, with complications including diabetes, cardiovascular disease and even cancer [48, 49]. Although the function of probiotics on metabolic syndrome has been reported [50, 51] the effect of probiotics on cardiovascular disease is unclear.

In the process of apoptosis in cardiac myocytes, cell gaps are produced and fibroblast recruitment takes place resulting in collagen accumulation and cardiac fibrosis leading to heart failure [52]. According to this progression, fibrosis is a key pathological phenomenon of heart failure. This study showed that a high-fat diet induced cardiac fibrosis in an obese animal model. Fibrosis-related transcription factor SP1 and protein marker MMP2 and TGF-β were up-regulated in HF group via Western blot assays. Cardiac fibrosis was confirmed using Masson's trichrome staining showing that the high-fat diet induced collagen accumulation (Fig. 1, white arrow). Treatment with multi-strain probiotics provides a protective function in high-fat diet-induced fibrosis by down-regulating the molecular pathway.

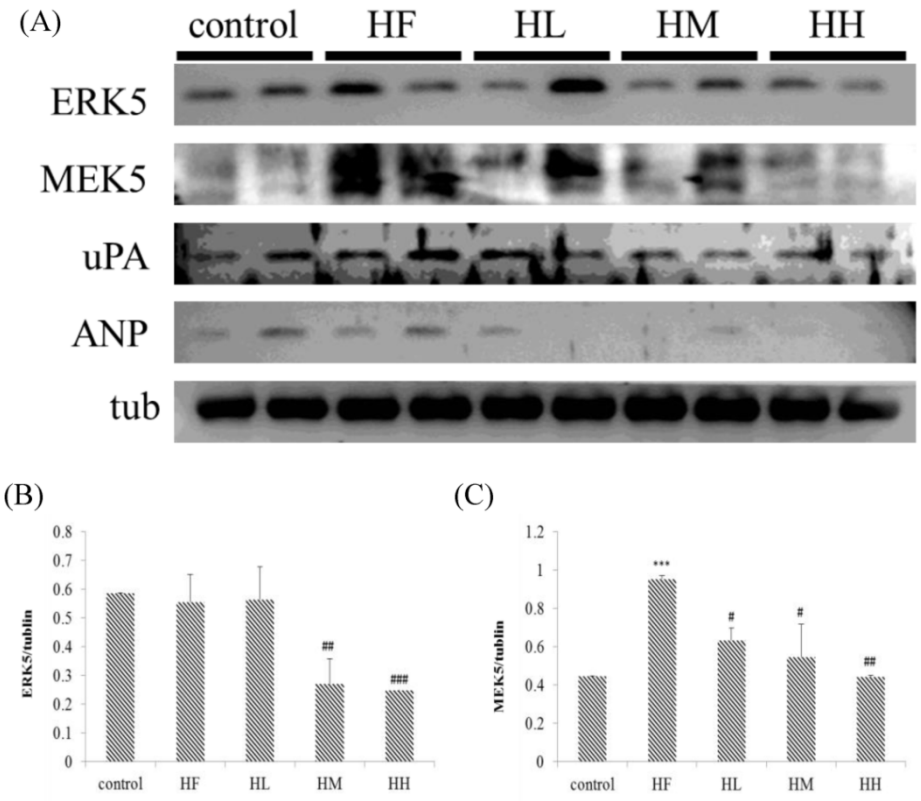

Figure 5. Hypertrophy-related protein expression analysis via Western blot. The autophagy-related protein levels were increased in the left ventricular tissue of high-calorie diet-induced obese rats, whereas treatment with different doses of multi-strain probiotics reduced these fibrosis-related proteins. (A) Western blots for the fibrosis pathway proteins. Quantitative analysis of the expression levels of (B) ERK5, (C) MEK5, (D) uPA and (E) ANP normalized to those of tubulin, #P<0.05, ##P<0.01 or ###P<0.001: the mean values were significantly different from those of the control group.

Other studies have noted that obesity may induce cardiac hypertrophy [53], but the mechanism underlying the promotion of hypertrophy is unknown. Our results indicated that a high-fat diet results in cardiac hypertrophy (Figure 1 and 2). However, treatment with multi-strain probiotics prevented high-fat diet-induced cardiac hypertrophy in all of the groups supplemented with different probiotic doses. Importantly, we determined the protein marker levels using Western blotting. The results indicated that high-fat diet cardiac eccentric hypertrophy was induced by activating ERK5 and MEK5, which are the key upstream markers in the eccentric hypertrophy pathway. Oral multi-strain probiotic administration prevented high-fat diet-induced cardiac eccentric hypertrophy by decreasing ERK5 and MEK5 expression. Moreover, a high-fat diet induced upstream protein expression of cardiac hypertrophy; however, no significant differences were observed between the control group and the HF group in the expression of downstream proteins ANP and uPA (Figure 5). Interestingly, supplementation with different doses of multi-strain probiotics decreased uPA and ANP expression. These findings suggested that oral multi-strain probiotics might improve a protective effect on cardiac hypertrophy in the hearts of rats fed a high-fat diet.

Autophagy is an important mechanism that regulates heart metabolism [54]. Previous research indicated that autophagy imbalance induced cell apoptosis in different organs [55-57]. We determined the expression of the important autophagy early stage-related protein markers LC3B and ATG7. A high-fat diet induces LC3B and ATG7 expression. After supplementation with low-, medium- and high doses of multi-strain probiotics, LC3B and ATG7 expression was significantly decreased. This result was confirmed with IHC staining. We obtained the same result that multi-strain probiotics decreased high-fat diet-induced LC3B expression in a dose-dependent manner. These data suggest a high-fat diet-induced autophagy pathway imbalance.

These results showed that a high-fat diet induced cardiac fibrosis, autophagy and hypertrophy. Our experiments showed that oral administration of multi-strain probiotics provided cardiac protection via regulation of fibrosis, autophagy and hypertrophy in the hearts of rats fed a high-fat diet.

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Competing Interests

The authors have declared that no competing interest exists.

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